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The major goal in the first year of the project is to investigate the *in vitro* anti-tumor activity of (-)-gossypol using prostate cancer cell lines. We have finished the tasks proposed in the Statement of Work for the first year. Specifically, we have investigated the *in vitro* anti-tumor activity of (-)-gossypol and potential synergistic effects of (-)-gossypol in combination with chemotherapeutic drugs. (-)-gossypol showed potent anti-tumor activity to human prostate cancer PC-3, LnCaP, CL-1 cells, but only limited or minimal effect on DU-145 and human normal prostate epithelial cells (PrEC) with low Bcl-xL. (-)-gossypol potently enhanced apoptosis induction by CDDP and docetaxel, currently used chemotherapeutic agents for prostate cancer. In PC-3 and CL-1 cells, (-)-gossypol showed either additive or more than additive effects in combination of CDDP and docetaxel using MTT-based WST-1 assay. (-)-gossypol potently enhanced X-ray irradiation induced growth inhibition in a clonogenic assay, and apoptosis induction in Annexin V and PI staining assays. The data obtained in the first year provide us a solid foundation to move the project to *in vivo* testing and further mechanism studies, to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of prostate cancer with Bcl-X_L overexpression.

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I. Introduction:

In this project, we will investigate in vitro and in vivo anti-tumor activity and the mechanism of action of (-)-gossypol in human prostate cancer with Bcl-X_L overexpression, and investigate the potential synergistic effects of (-)-gossypol in combination with chemotherapeutic drugs. Our basic hypothesis to be tested is that Bcl-X_L is the primary molecular target that mediate the anticancer activity of (-)-gossypol in human prostate cancer cells. Our ultimate goal is to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of prostate cancer with Bcl-X_L overexpression.

II. Research progress and key research acomplishments:

This project was started one year ago. During the first year of the project, we have finished the tasks proposed in the Statement of Work for the corresponding period of time. Specifically, we have finished the Task 1 (months 1-12): To investigate *in vitro* anti-tumor activity of (-)-gossypol and potential synergistic effects of (-)-gossypol in combination with chemotherapeutic drugs:

1. Analyze the in vitro cytotoxicity of (-)-gossypol to human prostate cancer cell lines (months 1-6): (-)-gossypol showed potent anti-tumor activity to human prostate cancer PC-3, LnCaP, CL-1 cells (with high levels of Bcl-xL, but only limited effect on DU-145 cells with low Bcl-xL, and minimal effect on human normal prostate epithelial cells (PrEC) and fibroblast cells WI-38 with undetectable Bcl-xL. (-)-gossypol potently blocked the prostate cancer cells in G1-phase in cell cycle analysis.
2. Evaluate the drug combination-induced apoptosis in human prostate cancer cell lines in vitro (months 4-9): (-)-gossypol potently enhanced apoptosis induction by CDDP and docetaxel, currently used chemotherapeutic agents for prostate cancer.
3. Analyze the effect of drug combination on cytotoxicity and apoptosis induction (months 6-12): In PC-3 and CL-1 cells, (-)-gossypol showed either additive or more than additive effects in combination of CDDP and docetaxel using MTT-based WST-1 assay. (-)-gossypol potently enhanced X-ray irradiation induced growth inhibition in a clonogenic assay, and apoptosis induction in Annexin V and PI staining assays.

III. Reportable outcomes:

1. One paper (1) published in the journal *Molecular Cancer Therapeutics* in 2005.

   This paper is supported in part by this PRCP grant and in part by a NIH NCI SPORE pilot grant.

   Liang Xu*, Dajun Yang, Shaomeng Wang, Wenhua Tang, Meilan Liu, Mary Davis, Jianyong Chen, James Rae, Theodore Lawrence and Marc Lippman. (-)-Gossypol Enhances Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer. *Mol Cancer Ther*, 2005, 4(2):197-205. (*Corresponding author)

2. One peer reviewed grant awarded in 2004:

Based on the data obtained from this PRCP grant, we applied and obtained a Pilot Project award from NIH NCI SPORE program in University of Michigan, working on (-)-gossypol and its derivatives in radiosensitization of human prostate cancer.

SPORE 2 P50 CA69568-06A1 (Program P.I.: K. Pienta) 10/01/04 – 9/31/05
NIH/NCI Pilot project in SPORE in Prostate Cancer $90,000
Potent Bcl-2/Bcl-X_L-bispecific Small Molecule Inhibitor, (-) Gossypol, as Novel Molecular Targeted Therapy for Hormone-Refractory Prostate Cancer
The major goal of the proposal is to test the activity of Gossypol alone or in combination with radiotherapy for treatment of prostate cancer.
Role: Principal Investigator in this pilot project.

3. One Program Project (U19) grant applied in 2004 and likely to be funded:

In 2004, we applied a U19 Program Project grant which consists of three R01 type Laboratory Programs. The U19 grant scored **131**, percentile **2.5%**, likely to be funded. I am a co-leader in the Laboratory Program #3 and some of the data was obtained from the PCRP grant.

1 U19 CA113317 (P.I.: Shaomeng Wang) 05/01/2005 – 04/30/2010
NIH NCI U19 Program grant $814,294/year
Novel small-molecule inhibitors of Bcl-2/Bcl-xL proteins
The major goal of the proposal is to discover and design more potent small molecule based modulators of the functions of Bcl-2/Bcl-xl proteins
Role: **Co-leader** in Laboratory Program #3 (with budget $257.087/year)

4. Three abstracts (2-4) funded from the PRCP grant were presented in international meetings. One abstract was selected for **Press Release** by the Organizing Committee in *EORTC-NCI-AACR Conference on "Molecular Targets and Cancer Therapeutics"* in Geneva, Switzerland, 2004. The PI, Dr. Xu, was interviewed by several press agencies during the meeting and there have been over 10 news reports published discussing the promising new therapy for prostate cancer funded by the PCRP grant. In the *International Conference on Tumor Progression and Therapeutic Resistance*, Dr. Xu was awarded the **2nd Prize of Poster Award.**

- Liang Xu, et al. Discovery and therapeutic potential of novel Bcl-2/Bcl-xL small-molecule inhibitors in human breast and prostate cancer. *International Conference on Tumor Progression and Therapeutic Resistance.* Philadelphia, PA, November 8-9, 2004. (Awarded **2nd Prize of Poster Award).**
- Liang Xu, et al. Radiosensitization of Human Prostate Cancer by Natural Polyphenol Inhibitor of Bcl-2/XL, (-)-Gossypol, Results in Tumor Regression. *EORTC-NCI-AACR Conference on "Molecular Targets and Cancer Therapeutics,"* Geneva, Switzerland, September 28-October 1, 2004. (Selected for **Press Release** by Organizing Committee).
- Liang Xu, et al. Gossypol(-), a Potent Small Molecule Inhibitor of Bcl-2/xl, Improves Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer. *The 95th American Association for Cancer Research Annual Meeting,* Orlando, Florida, March 27-31, 2004.

5. One investigational new drug (IND) application filed in 2004, pending FDA approval, on (-)-gossypol safety in human beings.

IV. Conclusions:

The major goal in the first year of the project is to investigate the *in vitro* anti-tumor activity of (-)-gossypol using prostate cancer cell lines. We have investigated the *in vitro* anti-tumor activity of (-)-gossypol and potential synergistic effects of (-)-gossypol in combination with chemotherapeutic drugs. (-)-gossypol showed potent anti-tumor activity to human prostate cancer PC-3, LnCaP, CL-1 cells, but only limited or minimal effect on DU-145 and human normal prostate epithelial cells (PrEC) with low Bcl-xL. (-)-gossypol potently enhanced apoptosis induction by CDDP and docetaxel, currently used chemotherapeutic agents for prostate cancer. In PC-3 and CL-1 cells, (-)-gossypol showed either additive or more than additive effects in combination of CDDP and docetaxel using MTT-based WST-1 assay. (-)-gossypol potently enhanced X-ray irradiation induced growth inhibition and apoptosis induction. The data obtained in the first year provide us a solid foundation to move the project to *in vivo* testing and further mechanism studies, to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of prostate cancer with Bcl-X_L overexpression.

V. References:

1. **Liang Xu***, Dajun Yang, Shaomeng Wang, Wenhua Tang, Meilan Liu, Mary Davis, Jianyong Chen, James Rae, Theodore Lawrence and Marc Lippman. (-)-Gossypol Enhances Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer. *Mol Cancer Ther*, 2005, **4**(2):197-205. (*Corresponding author)

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VI. APPENDICES:

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Liang Xu*, Dajun Yang, Shaomeng Wang, Wenhua Tang, Meilan Liu, Mary Davis, Jianyong Chen, James Rae, Theodore Lawrence and Marc Lippman. (-)-Gossypol Enhances Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer. *Mol Cancer Ther*, 2005, **4**(2):197-205. (*Corresponding author)

(−)-Gossypol enhances response to radiation therapy and results in tumor regression of human prostate cancer

Liang Xu,[1] Dajun Yang,[1] Shaomeng Wang,[1] Wenhua Tang,[1] Meilan Liu,[1] Mary Davis,[2] Jianyong Chen,[1] James M. Rae,[1] Theodore Lawrence,[2] and Marc E. Lippman[1]

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Abstract

Radioresistance markedly impairs the efficacy of tumor radiotherapy and involves antiapoptotic signal transduction pathways that prevent radiation-induced cell death. The majority of human prostate cancers overexpress the important antiapoptotic proteins Bcl-2 and/or Bcl-xL, which render tumors resistant to radiation therapy. (−)-Gossypol, a natural polyphenol product from cottonseed, has recently been identified as a potent small molecule inhibitor of both Bcl-2 and Bcl-xL. In the current study, we investigated the antitumor activity of (−)-gossypol in prostate cancer and tested our hypothesis that (−)-gossypol may improve prostate cancer's response to radiation by potentiating radiation-induced apoptosis and thus making cancer cells more sensitive to ionizing radiation. Our data show that (−)-gossypol potently enhanced radiation-induced apoptosis and growth inhibition of human prostate cancer PC-3 cells, which have a high level of Bcl-2/Bcl-xL proteins. Our *in vivo* studies using PC-3 xenograft models in nude mice show that orally given (−)-gossypol significantly enhanced the antitumor activity of X-ray irradiation, leading to tumor regression in the combination therapy. *In situ* terminal deoxynucleotidyl transferase–mediated nick end labeling staining showed that significantly more apoptotic cells were induced in the tumors treated with (−)-gossypol plus radiation than either treatment alone. Anti-CD31 immunohistochemical staining indicates that (−)-gossypol plus radiation significantly inhibited tumor angiogenesis. Our results show that the natural polyphenol inhibitor of Bcl-2/Bcl-xL, (−)-gossypol, can radiosensitize prostate cancer *in vitro* and *in vivo* without augmenting toxicity. (−)-Gossypol may improve the outcome of current prostate cancer radiotherapy and represents a promising novel anticancer regime for molecular targeted therapy of hormone-refractory prostate cancer with Bcl-2/Bcl-xL overexpression. [Mol Cancer Ther 2005;4(2):197–206]

Introduction

Radioresistance markedly impairs the efficacy of tumor radiotherapy and involves antiapoptotic signal transduction pathways that prevent radiation-induced cell death (1). Overexpression of the antiapoptotic protein Bcl-2 is observed in 30% to 60% of prostate cancer at diagnosis and in nearly 100% of hormone-refractory prostate cancer (2). Bcl-2 is significantly overexpressed in localized recurrent prostate carcinoma after radiation therapy, suggesting that alterations in the apoptotic pathway may be important in the development of local recurrence (3). Bcl-xL is found to be overexpressed in 80% to 100% of hormone-refractory prostate cancer and is associated with advanced disease, poor prognosis and shortened survival (4). Overexpression of Bcl-2/Bcl-xL antiapoptotic proteins decreases the proapoptotic response to such cellular insults as irradiation, chemotherapy, and androgen withdrawal, leading to resistance to the treatments (5). Thus, inhibition of the antiapoptotic functions of Bcl-2/Bcl-xL represents a novel and promising strategy for overcoming the resistance to current therapy for prostate cancer. Antisense Bcl-2 and Bcl-xL studies have provided important proof-of-the-concept that inhibition of Bcl-2 and Bcl-xL may be an effective new therapeutic strategy for the treatment of advanced prostate cancer (6–8). Recently, Wacheck et al. (9) showed that specific reduction of Bcl-xL protein levels by Bcl-xL antisense oligonucleotides indeed radiosensitized Caco-2 colon cancer cells which have a high level of Bcl-xL and are resistant to radiation. Scott et al. (10) showed that Bcl-2 antisense reduced prostate cancer cell survival following irradiation. Nonpeptide, small-molecule inhibitors of Bcl-2 and Bcl-xL have several major advantages over Bcl-2/Bcl-xL antisense oligonucleotides, antibodies, or peptides, including better bioavailability, better *in vivo* stability, lower cost, and oral activity (11).

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The potential antitumor and chemoprevention activities of some natural polyphenol compounds have been linked to their direct inhibition of antiapoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL (12). (−)-Gossypol, a natural polyphenol product from cottonseed, has recently been identified as a small-molecule inhibitor of both Bcl-2 and Bcl-xL and potently induces apoptosis in several cancer cell lines (12–14). Because the majority of human prostate cancers overexpress Bcl-2 and/or Bcl-xL, rendering the tumors resistant to radiation therapy, we investigated whether (−)-gossypol can potentiate prostate cancer's response to radiation and whether this potentiation is accompanied by an increase of radiation-induced apoptosis. Our results show that (−)-gossypol significantly enhances the antitumor activity of radiation therapy *in vitro* and *in vivo* without augmenting toxicity and represents a promising new anticancer regime for molecular targeted therapy of hormone-refractory prostate cancer with Bcl-2/Bcl-xL overexpression.

Materials and Methods

Cell Culture and Reagents

All cell lines used were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely maintained in an improved minimal essential medium (Biofluids, Rockville, MD) with 10% fetal bovine serum and 2 mmol/L L-glutamine. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2. (−)-Gossypol was produced at RTI International (Research Triangle Park, NC) under a National Cancer Institute (NCI) contract to support the RAID Project (D. Yang). For *in vitro* experiments, (−)-gossypol was dissolved in DMSO at 20 mmol/L as stock solution. For *in vivo* studies, (−)-gossypol was made fresh before each administration. It was first dissolved in ethanol and then diluted with sterile water within 5 minutes, to a final ethanol concentration of 10%.

Radiation Clonogenic Assay

To investigate the effect of (−)-gossypol on cancer cells' response to radiation, a standard clonogenic assay was done as described with modification (15, 16). Briefly, 200 to 10,000 PC-3 cells per well in 6-well plates were treated with 1 to 5 μmol/L (−)-gossypol, and the cells were irradiated within one hour with 2 to 8 Gy using 300 kV X-rays. One-milliliter complete medium was added per well in the 6-well plates on day 5. After another 5 to 7 day's culture, the plates were stained with crystal violet, and the colonies with over 50 cells were counted with a ColCount colony counter (Oxford Optronix, Oxford, United Kingdom). The same amount of solvent DMSO was added in control wells as vehicle control. For each combination treatment, parallel analyses with each agent alone were also done. The cell survival curves were plotted using a linear-quadratic model, and the mean inactivation dose (area under the cell survival curve) was calculated as described previously (17). The cell survival enhancement ratio was calculated as the ratio of the mean inactivation dose in control divided by the mean inactivation dose in treated cells (18).

Apoptosis Assay

For apoptosis analysis by flow cytometry, PC-3 cells were treated with (−)-gossypol and X-ray irradiation, alone or in combination, in 12-well plates as indicated in Fig. 2 legend, then trypsinized and washed with PBS, and fixed in 70% ethanol on ice. After centrifugation, cells were stained with 50 μg/mL propidium iodide and 0.1 μg/mL RNase A and analyzed by flow cytometry using a FACStar Plus cell sorter. Each histogram was constructed with the data from at least 5,000 events. Data were analyzed to calculate the percentage of sub-G_1 population (apoptosis) using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Animal Model and *In vivo* Experiments

Male athymic NCr-*nu/nu* nude mice ages 5 to 6 weeks were purchased from NCI. Mice were inoculated s.c. on both sides of the lower back above the tail after alcohol preparation of the skin, using a sterile 22-gauge needle with 0.1 mL of cell suspension of 5×10^6 PC-3 cells with manual restraint. When tumors reached appropriate size, as indicated in the results figures, the mice were randomized into five to eight mice per group and treated with either (−)-gossypol 10 mg/kg p.o. q.d. ×5 × 4 weeks, or X-ray irradiation 2 Gy, q.d. ×5, × 3 weeks, or the combination of (−)-gossypol and radiation. The vehicle control group and the radiation-only group received the same amount of 10% ethanol which is the solvent for (−)-gossypol. The tumor sizes and animal body weights were measured twice a week by a technician from University of Michigan Unit of Laboratory Animal Medicine without knowledge of the treatment. All animal experiments were done according to the protocol approved by University of Michigan Guideline for Use and Care of Animals.

Tumor Apoptosis and Angiogenesis Analysis

Tumors from each group were excised and stained for apoptosis by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining using the ApopTag kit. This method is very sensitive and allows for determination of very few apoptotic cells *in situ*. Tumor angiogenesis was analyzed by anti-mouse CD31 immunohistologic staining of tumor sections.

X-ray Irradiation

A 300-kV X-ray irradiator in the University of Michigan Radiation Core Facility was employed at a dose rate of ~3 Gy/min. Dosimetry was carried out using an ionization chamber connected to an electrometer system which is directly traceable to a National Institute of Standard and Technology Calibration. For *in vivo* irradiation, mice were restrained such that only the tumor areas were exposed to irradiation, whereas the rest of the animal body was shielded, as described previously (19–21). A fractionated dose schedule of 2 Gy q.d. ×5 for 3 weeks was employed, which is consistent with a clinical radiotherapy regime for prostate cancer. Also, we had previously used this regimen successfully for radiation in combination with p53 gene therapy and antisense therapy (19, 21–23). Control groups and (−)-gossypol only groups animals were also put in restrainers for ~2 minutes as a sham irradiation.

Statistical Analysis

Two-way ANOVA and two-tailed *t* tests were employed to analyze the animal data using Prism 3.0 software (Graphpad Prism, San Diego, CA). The synergism analysis for the combination effects of (−)-gossypol and radiation was analyzed by Chou-Talalay's combination index-isobologram and multiple drug dose-effect analysis method (24) using CalcuSyn software (Biosoft, Inc., Cambridge, United Kingdom).

Results

(−)-Gossypol Sensitizes PC-3 Cells to X-ray Irradiation in an *In vitro* Clonogenic Assay

Treatment of PC-3 cells with (−)-gossypol significantly reduced the radiation resistance of PC-3, resulting in 10- and 20-fold reductions of colony formation from control at 6 and 8 Gy, respectively (Fig. 1). Table 1 summarizes the radiation response characteristics from Fig. 1. (−)-Gossypol-mediated radiosensitization correlated with (−)-gossypol doses ($r = 0.95$, $P = 0.0028$). Similar correlations were also observed with other radiation response variables as shown in Table 1. We also analyzed the interaction between (−)-gossypol and radiation using Chou-Talalay's synergism analysis, a widely used method to analyze and quantify the synergy in combination therapies (24–28). As shown in Fig. 1A and B, treatment of PC-3 cells with radiation plus nontoxic doses (3 and 4 μmol/L) of (−)-gossypol achieved the combination index 0.68 and 0.34, dose reduction index 1.9 and 4.3, respectively. Combination index <1 and dose reduction index >1 indicates synergy between the two treatments (29, 30). (−)-Gossypol (5 μmol/L) showed better synergy with radiation, but this dose of (−)-gossypol was cytotoxic alone (Table 1; Fig. 1A). The results show that the combination of (−)-gossypol with radiation resulted in significant synergy in inhibiting clonogenic cell survival of PC-3 cells. (−)-Gossypol can sensitize PC-3 cells to X-ray irradiation in a dose-dependent manner.

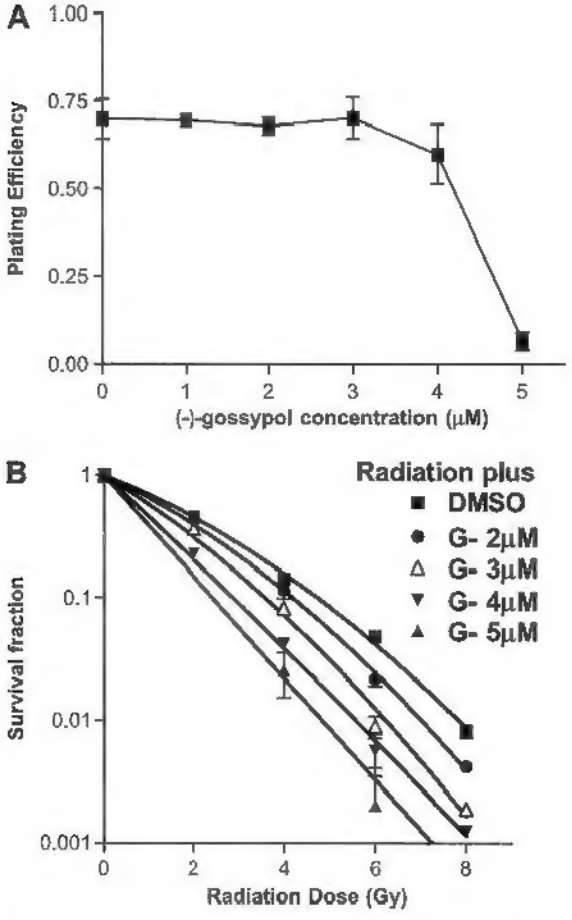

Figure 1. Clonogenic assay of PC-3 cells exposed to X-ray irradiation with or without (−)-gossypol [*G(−)*]. PC-3 cells were plated in 6-well plates and G(−) or DMSO vehicle control were added. Then cells were irradiated with X-ray, 300 kV, dose rate ~3 Gy/min at room temperature with doses up to 8 Gy. The plates were cultured for 12 d, stained with crystal violet, and the colonies with over 50 cells were counted with ColCount colony counter. The survival curves were plotted with linear-quadratic model. **A,** plating efficiency (survival fraction) of G(−) treatment alone. **B,** clonogenic survival curves of PC-3 cells in response to irradiation [normalized for the killing of G(−)].

(−)-Gossypol Enhances Radiation-Induced Apoptosis in PC-3 Cells

As shown in Fig. 2A and B, (−)-gossypol and X-ray irradiation potently induced apoptosis in PC-3 cells in a dose- and time-dependent manner, with a plateau around 60% apoptosis. Similar results were observed with Annexin V staining for early apoptotic cells (data not shown). Based on these data, treatment conditions that induced apoptosis in the linear range (e.g., 6 Gy radiation dose and 10 μmol/L (−)-gossypol dose at 48 hours post-irradiation), were chosen in subsequent combination studies. Fig. 2C shows that (−)-gossypol significantly enhanced radiation-induced apoptosis when added to the cells 24 hours after irradiation. In this experiment, PC-3 cells were irradiated first, then

Table 1. Effects of (−)-gossypol on X-ray radiation dose-response of PC-3 cells in clonogenic assay

(−)-Gossypol (μmol/L)	MID	E ratio	Gy (1%)	SF (2 Gy)	CI	DRI
0	2.22	1	7.84	0.45	—	—
1	2.06	1.08	7.11	0.43	0.78	1.48
2	1.95	1.14	7.03	0.4	0.8	1.5
3	1.63	1.36	6.25	0.31	0.68	1.9
4	1.26	1.76	5.59	0.21	0.34	4.3
5	1.05	2.11	4.84	0.15	0.27	6.1

NOTE: Radiobiological parameters calculated from survival curves in Fig. 1 based on linear-quadratic model. The synergism analysis of (−)-gossypol with radiation at 8 Gy were performed with Chou-Talalay's combination index-isobologram and multiple drug-dose effect analysis method as described in Materials and Methods. CI < 1, DRI >: synergy between the two treatments.

Abbreviations: MID, mean inactivation dose; E ratio, enhancement ratio calculated from MID in control cells divided by MID in treated cells; Gy (1%), radiation dose leading to 1% cell survival; SF (2 Gy), survival fraction at 2 Gy; CI, combination index; DRI, dose reduction index.

(−)-gossypol was added 24 hours later, cells were collected 48 hours after radiation for analysis of apoptosis. Isobologram synergism analysis (ref. 30; Fig. 2D) shows that, under this condition, 6 Gy radiation and 10 μmol/L (−)-gossypol treatment resulted in more than additive induction of apoptosis, as compared with either treatment alone, with combination index = 0.345, indicating a strong synergy in the combination therapy (30); 6 Gy radiation and 5 μmol/L (−)-gossypol treatment resulted in only an additive effect (combination index = 0.963). Interestingly, treating cells with radiation and (−)-gossypol at the same time or with (−)-gossypol 24 hours before radiation did not show any significant potentiation (Fig. 2E and F), suggesting that (−)-gossypol-mediated radiation potentiation is combination sequence specific. Our data are consistent with that of Kruit et al. (31), in which treatment with doxorubicin for 24, 48, and 72 hours after irradiation potentiated irradiation cytotoxicity of PC-3 cells. This combination sequence effect may be related to cell cycle of PC-3 cells where cells at different phases have different response to radiation and (−)-gossypol, as suggested in other cell or drug models (13, 17, 32–34).

(−)-Gossypol Enhances Prostate Cancer Response to Radiation and Results in Tumor Regression *In vivo*

To investigate whether (−)-gossypol can sensitize PC-3 cells to radiation *in vivo*, we employed a PC-3 xenograft model in athymic nude mice. Three independent *in vivo* experiments with different initial tumor sizes were carried out and data are shown in Fig. 3. As shown in Fig. 3A, with an initial tumor size of ~100 mm^3 at the start of irradiation, (−)-gossypol inhibited PC-3 tumor growth *in vivo* in the first 2 weeks of therapy (versus Vehicle control, $P < 0.05$, Student's t test, $n = 16$), as did X-ray irradiation alone, but the tumors quickly grew back in the third week and no tumor regression was observed. However, the combination of (−)-gossypol and radiation showed significantly improved antitumor activity and was more effective than either treatment alone ($P < 0.01$ versus radiation and $P < 0.001$ versus (−)-gossypol alone, $n = 16$). More importantly, combination therapy resulted in tumor regression in all the xenografts in the fourth week, to 24% of their peak tumor sizes (46 ± 40 mm^3 on day 30 versus 191 ± 140 mm^3 on day 16). It is worth noting that only the combination therapy resulted in >50% tumor regression,

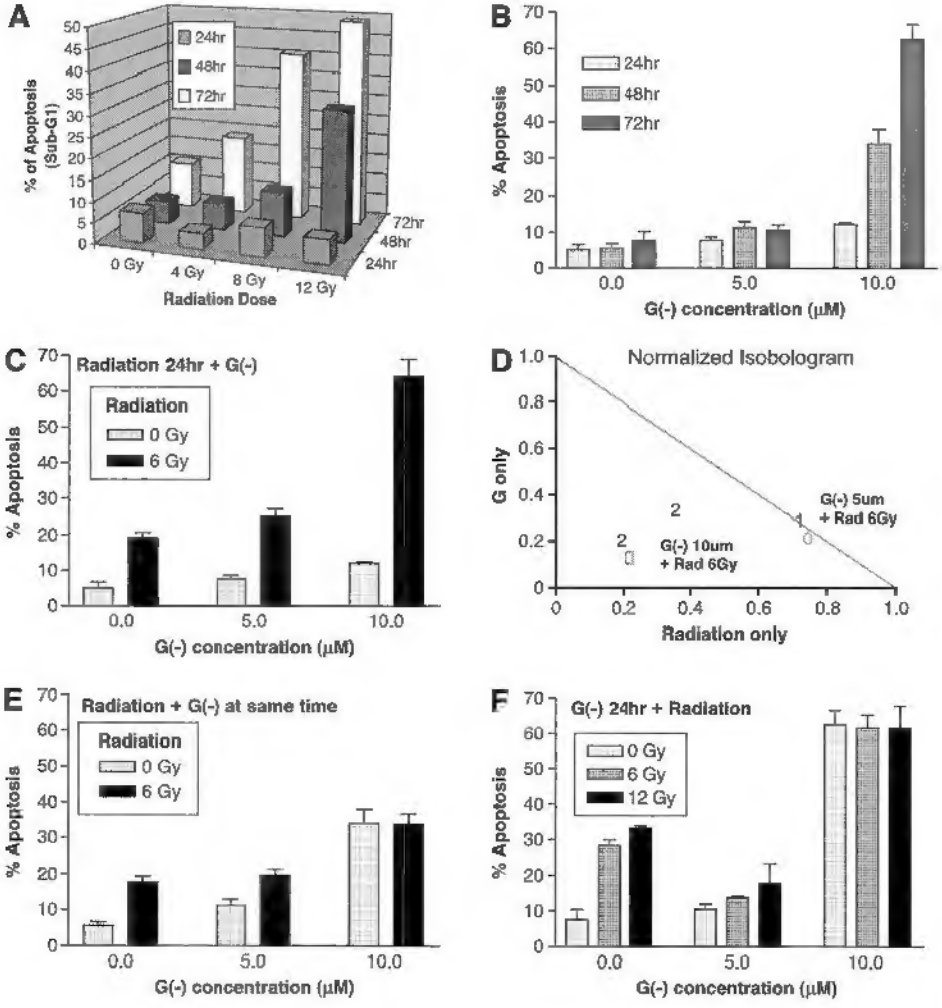

Figure 2. (−)-Gossypol [*G(−)*] potentiates radiation-induced apoptosis of PC-3 cells. PC-3 cells were plated in 12-well plates overnight, and treated as indicated. At indicated time after treatment, the cells were trypsinized and fixed in 70% ethanol on ice, stained with PI, and analyzed by flow cytometry. The percentage of apoptotic cells is the percent of cells with sub-G_1 nuclei staining ($n = 2$). Representative results of at least three independent experiments. **A,** X-ray radiation-induced apoptosis is dose and time dependent. Cells were treated at indicated doses for 24, 48, and 72 h. **B,** G(−)-induced apoptosis of PC-3 cells. G(−) potently induced apoptosis at 10 μmol/L. **C,** G(−) enhanced radiation-induced apoptosis when added to the cells 24 h after irradiation. Cells were irradiated first, added G(−) 24 h later, and cells collected 48 h after radiation. **D,** normalized isobologram of data in **C**. *1*, 6 Gy radiation + G(−) 5 μmol/L, combination index = 0.963, additive effect. *2*, 6 Gy radiation + G(−) 10 μmol/L, combination index = 0.345, synergy. Potentiation was not observed in the cells treated with G(−) and radiation at the same time (**E**), nor in the cells treated with G(−) first, and radiation 24 h later (**F**). Cells were collected 48 h after radiation for analysis.

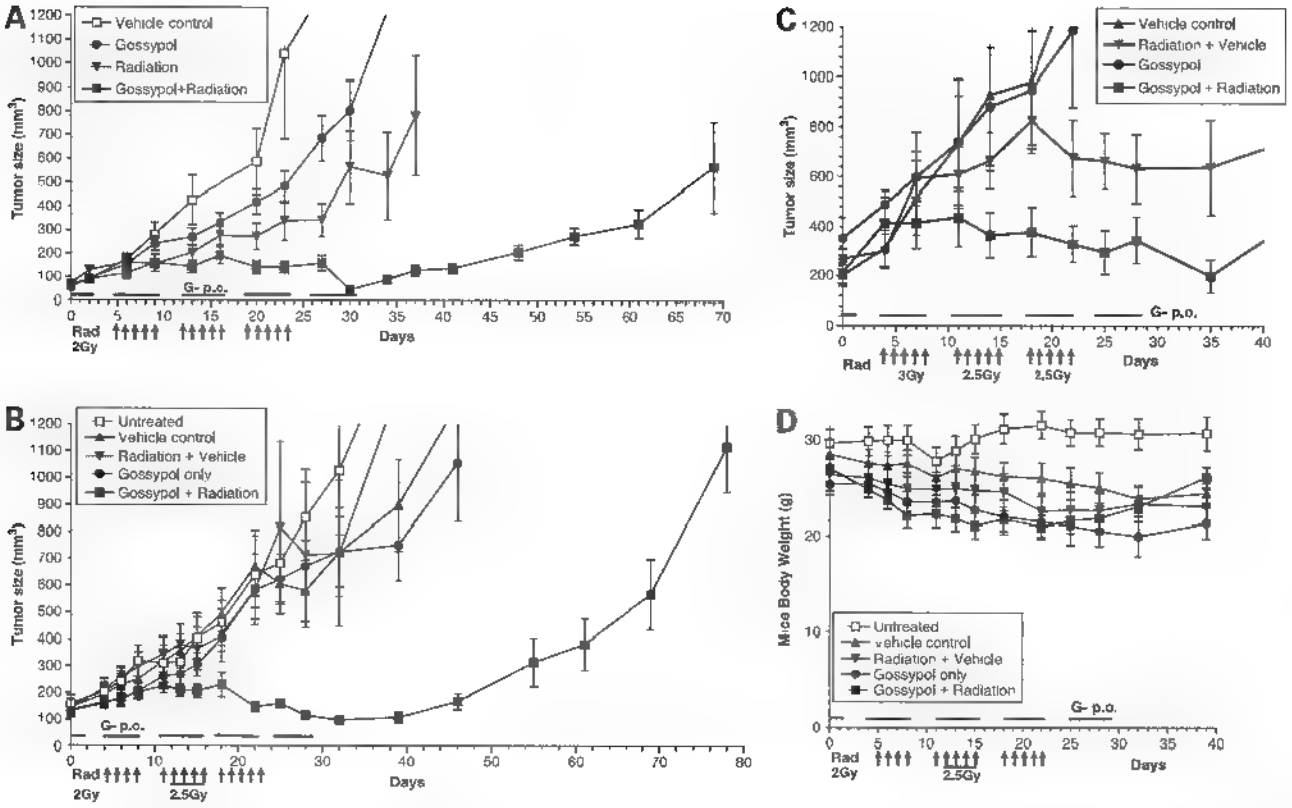

Figure 3. (−)-Gossypol [(−)G] in combination with radiation achieves tumor regression in androgen-independent prostate cancer PC-3 xenograft model PC-3 xenograft tumor growth curves after treatment with fractionated X-ray irradiation and (−)G, alone or in combination, where the tumor sizes at the start of (−)G treatment are around 75 mm^3 (**A**), 150 mm^3 (**B**), and 230 mm^3 (**C**). PC-3 cells (5×10^6) were s.c. injected into the lower back above the tail of each mouse, both sides. When tumors reached appropriate size, the mice were randomized into five to eight mice per group and treated with either (−)G 10 mg/kg p.o., q.d. ×5, ×4 weeks; or X-ray irradiation 2 to 3 Gy, q.d. ×5, ×3 weeks, or their combination. Vehicle control group and radiation only group received same amount of 10% ethanol which is solvent for (−)G. The average tumor sizes were shown ($n = 10-16$). The total radiation doses were 30 Gy for **A** and **B**, and 40 Gy for **C**. **D**, mice body weights from experiment B during treatment were plotted. (−)-Gossypol in combination with radiation seems not to have significant toxicity as compared with either treatment alone or vehicle control.

a criterion for clinical response to treatment, whereas the tumors in either (−)-gossypol alone group or radiation alone group progressed during treatment (although slower than control).

To investigate whether such improved antitumor efficacy can also be achieved with bigger tumors, we doubled the initial tumor size to ~200 mm^3 at the start of radiation treatment (Fig. 3B). Using the same dose schedule, (−)-gossypol or radiation alone had no obvious effect on these established large tumors, only the combination of (−)-gossypol and radiation achieved significant antitumor activity with >50% tumor regression ($P = 0.039$, versus radiation alone; and $P = 0.0003$, versus (−)-gossypol alone; $n = 8-10$). The tumor growth delay (T/C days) was 8.5 days for radiation alone, 0 days for (−)-gossypol alone and 54.5 days for combination of (−)-gossypol and radiation. The later effect is considered significant according to NCI criteria (35).

We further doubled the initial tumor size to ~400 mm^3 in our third *in vivo* experiment (Fig. 3C) in which the radiation dose was increased to 3 Gy q.d. ×5 for the first week and 2.5 Gy q.d. ×5 for the second and third weeks (total 40 Gy), with the same dose schedule of oral (−)-gossypol. As expected, a similar but profound antitumor effect was observed only in the mice treated with combination of (−)-gossypol and radiation, whereas neither treatment alone was active in these well-established large tumors (Fig. 3C). Importantly, the combination therapy was well tolerated by the animals; no significant animal body weight loss was observed (Fig. 3D) and no obvious organ toxicities were seen, suggesting the combination therapy did not augment toxicities.

The tumor growth inhibition (T/C %) values for these three *in vivo* studies were calculated as described (35) and summarized in Table 2. According to NCI criteria, T/C < 42% is considered significant antitumor activity, T/C < 10% is considered to indicate highly significant antitumor activity and is the level used by NCI to justify a clinical trial (DN-2 level activity; ref. 35). Combination of (−)-gossypol and radiation can achieve T/C up to 3.4%, or 96.6% inhibition in median tumor size, without significant toxicity, which meets NCI's DN-2 level activity (35).

(−)-Gossypol Increases Radiation-Induced Apoptosis of PC-3 Tumors and Inhibits Tumor Angiogenesis *In vivo*

To begin to address the question of why the combination of (−)-gossypol and radiation had such a profound efficacy versus either treatment alone, we took the tumor tissues from each group in animal experiment B (Fig. 3B) at the end of fractionated radiation (week 3) and did TUNEL staining for apoptosis and anti-mouse CD31 immunohistochemical staining for angiogenesis. TUNEL assay results are shown in Fig. 4A, the brown nucleic staining indicates TUNEL-positive apoptotic cells. (−)-Gossypol plus radiation induced significantly more apoptosis than either (−)-gossypol or radiation alone. Figure 4B(a) shows the increased apoptotic cells were only seen in combination therapy treated tumor tissue but not in surrounding normal tissues.

Because the tumors in (−)-gossypol + Rad group were smaller than that in other groups, to exclude the influence of tumor size on the apoptosis results, we selected extra nude mice with matching tumor size (~400 mm^3) and gave oral (−)-gossypol 10 mg/kg q.d. for 3 days and a single dose of 10 to 20 Gy X-ray irradiation on day 3, or either treatment alone. The tumors were taken on day 4 (e.g., 24 hours after radiation and last dose of (−)-gossypol) for TUNEL staining. As shown in Fig. 4B, significant induction of apoptosis was observed in tumors treated with (−)-gossypol + 10 Gy radiation, whereas surrounding normal tissues were not affected by this irradiation dose (Fig. 4B(b)). On the contrary, 20 Gy radiation alone induced limited apoptosis in tumor tissue but much more apoptosis in surrounding normal tissues, (Fig. 4B(c–d)), consistent with the report that PC-3 is resistant to ionizing irradiation (36). Neither 10 Gy radiation alone nor (−)-gossypol alone induced significant apoptosis in tumor tissue (data not shown).

More strikingly, anti-mouse CD31 staining indicates that the combination of (−)-gossypol and radiation almost completely inhibited tumor angiogenesis, whereas either (−)-gossypol or radiation alone had limited effect on tumor blood vessel growth (Fig. 4C). We observed no significant difference in cell proliferation marker Ki-67 staining of these tumors (data not shown). The CD31 data strongly suggest that antiangiogenesis might be one of the mechanisms involved in the *in vivo* antitumor activity of (−)-gossypol in combination with radiation.

Discussion

Radiation therapy is used to treat all stages of localized prostate cancer (36). However, both clinical and radiobiological evidence indicate that prostate cancer cells can be relatively resistant to radiation (37, 38). PC-3 is a hormone-refractory human prostate cancer cell line, which is resistant to current chemotherapy and radiation therapy (36). PC-3 has very high levels of Bcl-2 and Bcl-xL expression that may contribute to PC-3's resistance to apoptosis induced by chemotherapy/radiotherapy (38). X-ray irradiation is known to be a strong inducer of apoptosis (36). Bcl-2/Bcl-xL overexpression protects cells from radiation-induced apoptosis and thus may play a role in PC-3 cell's radiation resistance. Rosser et al. reported that patients undergoing radical prostatectomy after radiotherapy had a significantly higher rate of Bcl-2 overexpression than did patients who underwent surgery as the initial treatment (3). Bcl-2 is overexpressed in localized recurrent prostate carcinoma after radiation therapy and is reported to be important in the development of local recurrence (3). Wacheck et al. (9) showed that specific reduction of Bcl-xL protein levels by Bcl-xL antisense oligonucleotides was able to radiosensitize Caco-2 colon cancer cells which have a high level of Bcl-xL. It would thus be of clinical interest to investigate whether blocking the antiapoptosis function of Bcl-2/Bcl-xL will overcome the radiation resistance and sensitize the cancer cells to standard radiation therapy.

The natural polyphenol compound (−)-gossypol has been shown to have antiproliferative and antimetastatic effects on many kinds of cancer (13, 39–42). Multiple modes of action and molecular targets have been proposed for the antitumor activity of gossypol (14, 39, 41, 43–46), including inhibition of protein kinase C activity (47), modulation of cell cycle regulatory proteins Rb and cyclin D1 (32), inhibition of cellular energy metabolism (48), direct toxicity to mitochondria (49), or antiangiogenesis (45), etc. Recently, (−)-gossypol has been reported as a potent small molecule inhibitor of both Bcl-2 and Bcl-xL and potently induces apoptosis in several cancer cell lines with high levels of Bcl-2/Bcl-xL (12, 14). These studies agreed that (−)-gossypol is a potent inducer of apoptosis in cancer cells and is well tolerated, clinically safe. More recently, Oliver et al. have shown that (−)-gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-xL-mediated apoptosis resistance, confirming that (−)-gossypol is a potent and novel therapeutic able to overcome apoptosis resistance by specifically targeting the activity of anti-apoptotic Bcl-2 family members (50). Our collaborators, Mohammad et al., have recently shown that (−)-gossypol has a significant antitumor activity *in vitro* and *in vivo* as a potential novel therapy for the treatment of lymphoma (51) and Oliver et al. showed that (−)-gossypol has potent antitumor activity in head and neck cancer cells *in vitro*, possibly by direct inhibition of Bcl-xL and Bcl-2 (50).

In the current study, we employed (−)-gossypol to investigate whether (−)-gossypol can potentiate prostate cancer's response to radiation and whether this potentiation is accompanied with increased radiation-induced apoptosis. Our data show that (−)-gossypol potently enhanced radiation-induced apoptosis and growth inhibition of human prostate cancer PC-3 cells. More importantly, (−)-gossypol significantly improved antitumor activity of X-ray irradiation to PC-3 cells *in vivo* in an established xenograft model of PC-3 tumors in nude mice, resulting in tumor regression even in large tumors. NCI DN-2 level activity could be achieved only with the combination of (−)-gossypol and radiotherapy. This enhanced response to radiation was accompanied by increased induction of apoptosis *in vivo* by the combination therapy. This is the first report demonstrating a potential

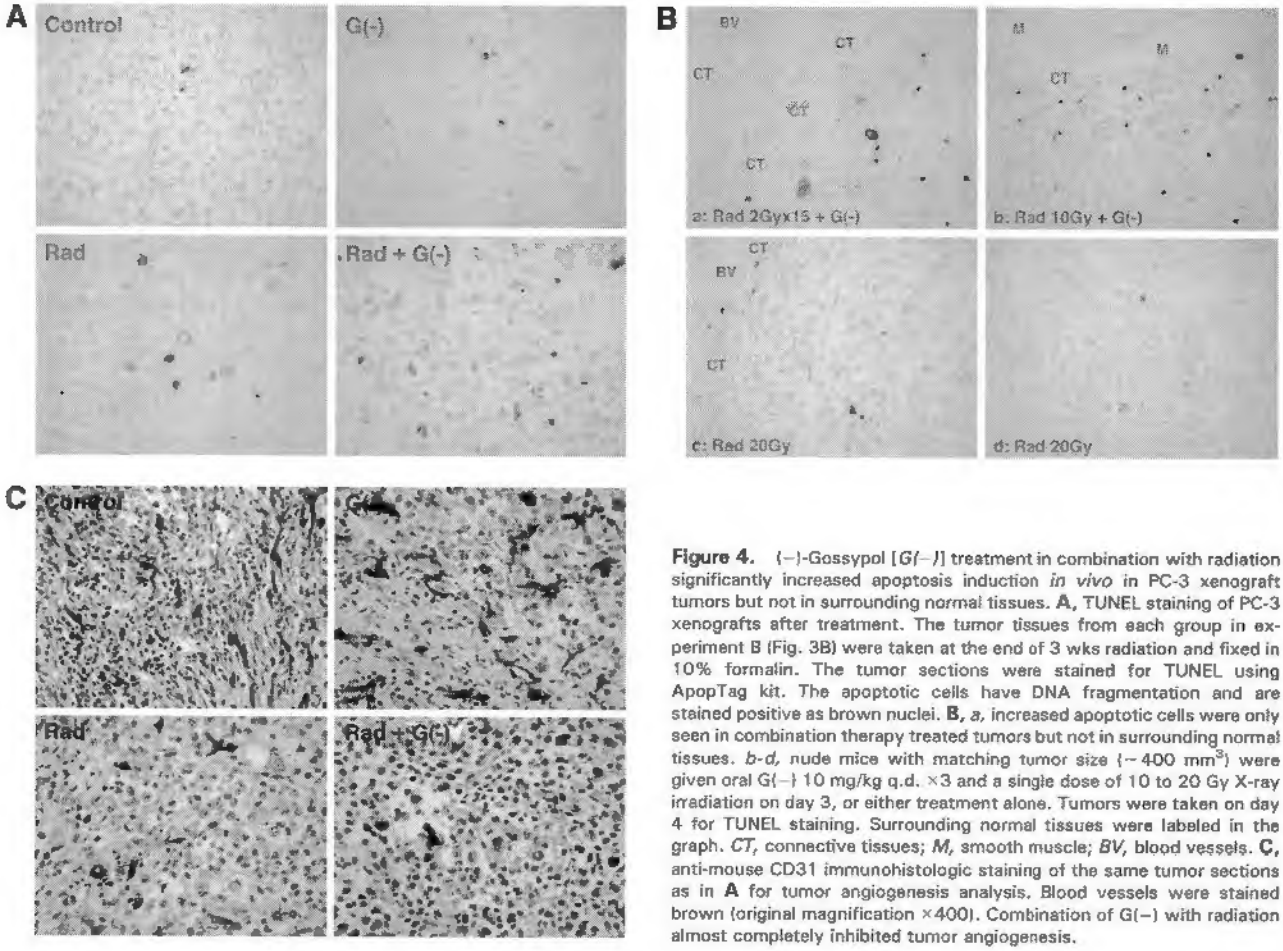

Figure 4. (−)-Gossypol [*G(−)*] treatment in combination with radiation significantly increased apoptosis induction *in vivo* in PC-3 xenograft tumors but not in surrounding normal tissues. **A,** TUNEL staining of PC-3 xenografts after treatment. The tumor tissues from each group in experiment B (Fig. 3B) were taken at the end of 3 wks radiation and fixed in 10% formalin. The tumor sections were stained for TUNEL using ApopTag kit. The apoptotic cells have DNA fragmentation and are stained positive as brown nuclei. **B,** *a,* increased apoptotic cells were only seen in combination therapy treated tumors but not in surrounding normal tissues. *b-d,* nude mice with matching tumor size (~400 mm^3) were given oral G(−) 10 mg/kg q.d. ×3 and a single dose of 10 to 20 Gy X-ray irradiation on day 3, or either treatment alone. Tumors were taken on day 4 for TUNEL staining. Surrounding normal tissues were labeled in the graph. *CT,* connective tissues; *M,* smooth muscle; *BV,* blood vessels. **C,** anti-mouse CD31 immunohistologic staining of the same tumor sections as in **A** for tumor angiogenesis analysis. Blood vessels were stained brown (original magnification ×400). Combination of G(−) with radiation almost completely inhibited tumor angiogenesis.

small molecule inhibitor of Bcl-2/Bcl-xL improves antitumor efficacy of radiation therapy both *in vitro* and *in vivo* with increased induction of apoptosis. Further studies are needed to establish a formal link between the observed efficacy of (−)-gossypol and its cellular target(s), as well as the role of apoptosis in (−)-gossypol-mediated radiosensitization.

The role of Bcl-2/Bcl-xL and apoptosis in radiosensitivity of cancer cells is cell type specific and cellular context dependant (1). Using a Bcl-2-transfected PC-3 cell clone, Kyprianou et al. (52) showed that Bcl-2 overexpression afforded a 3-fold protection from radiation-induced apoptosis, without affecting the clonogenic survival of human prostate cancer cells. However, these Bcl-2-transfected PC-3 cells may not depend on exogenous Bcl-2 for survival; thus, the transfected Bcl-2 may not have a direct effect on clonogenic survival of these transfected cells. Scott et al. (10) showed that down-regulation of endogenous Bcl-2 using Bcl-2 antisense reduced prostate cancer LnCap cell survival following irradiation, suggesting a potentially important therapeutic approach for enhancing radiosensitivity in prostate tumors via antisense oligonucleotide or other drug therapies that down-regulate Bcl-2. Our results show that (−)-gossypol, a reported small molecule functional inhibitor of Bcl-2/Bcl-xL (12, 14), potently radiosensitizes PC-3 cells and enhances radiation-induced apoptosis, both *in vitro* and *in vivo*. Our data are consistent with those reported results of Bcl-2/Bcl-xL antisense therapies (9, 10, 53–57) or Bcl-2 RNA interference (58).

Table 2. Comparison of tumor growth inhibition (T/C %) versus starting tumor sizes in the three *in vivo* experiments

Experiment*	Tumor sizes at start of radiation (mm^3)	Treatment		
		Rad [T/C(%)]	G(−) [T/C(%)]	Rad + G(−) [T/C(%)]
A	~100	36.9	95.8	3.4
B	~200	77.7	8.6	12.6
C	~300	43.0	~100	17.1

*Data based on *in vivo* experiments shown in Fig. 3A, B, and C.
NOTE: When the median tumor size in control group reached 750 mm^3, tumor growth inhibition (T/C %) were calculated as the % median tumor size in treatment group versus that in control group.
Abbreviations: Rad, radiation; G(−), (−)-gossypol.

It remains to be delineated whether (−)-gossypol-mediated radiosensitization is through inhibition of Bcl-2/Bcl-xL or other molecular targets. Due to the multi-target nature of (−)-gossypol, Bcl-2/Bcl-xL may not be the only cellular target(s) inhibited by (−)-gossypol. However, our recent studies using live-cell fluorescence resonance energy transfer and a coimmunoprecipitation pulldown assay indicate that (−)-gossypol potently blocks heterodimerization of Bcl-xL with Bax, Bad, and Bim at the (−)-gossypol doses that induce apoptosis and growth inhibition in prostate cancer cells.[3] In FL5.12-Bcl-xL cells, (−)-gossypol seems to be able to overcome the Bcl-xL-mediated protection against apoptosis induced by IL-3 withdrawal at a nontoxic dose of (−)-gossypol.[4] Our collaborators, Oliver et al., have recently showed an inverse correlation between Bcl-xL/Bcl-xS ratios and sensitivity to (−)-gossypol in head and neck cancer cell lines (59). Importantly, the head and neck cancer cell lines resistant to cisplatin were very sensitive to (−)-gossypol (59). Oliver et al. have also showed that (−)-gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-xL-mediated apoptosis resistance in Bcl-2/Bcl-xL-transfected Jurkat T cells (50). These recent data strongly support that Bcl-2/Bcl-xL may very likely be the major molecular target(s), if not the only target, in (−)-gossypol-induced apoptosis. We are currently pursuing detailed mechanistic studies to investigate molecular mechanism(s) of action of (−)-gossypol in radiosensitization of prostate cancer cells.

Several recent studies showed that Bcl-2 overexpression increases angiogenic potential of cancer cells through increasing angiogenic factors such as vascular endothelial growth factor, and treatment of cancer cells with a Bcl-2/Bcl-xL antisense oligonucleotide induces antiangiogenic activity via a reduction of hypoxia-induced vascular endothelial growth factor secretion (60, 61). Our CD31 staining data are consistent with these findings and strongly suggest that antiangiogenesis might be one of the mechanisms involved in the *in vivo* antitumor activity of (−)-gossypol in combination with radiation, possibly through inhibition of Bcl-2-mediated angiogenesis by (−)-gossypol. This observation sheds light on why only moderate radiosensitization effect was observed *in vitro* but a profound improvement of antitumor activity with tumor regression was seen *in vivo*.

In conclusion, our study shows that (−)-gossypol significantly enhances the antitumor activity of ionizing irradiation which is accompanied by increased induction of apoptosis, both *in vitro* and *in vivo*, and represents a promising novel anticancer regime for molecular targeted radiosensitization of human prostate cancer. Our data warrant further preclinical and clinical study of (−)-gossypol in combination with radiation therapy (and potentially other therapies) as a novel treatment modality for hormone-refractory prostate cancer with high levels of Bcl-2/Bcl-xL.

[3] S. Wang, in preparation.
[4] L. Xu, in press.

Acknowledgments

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References

1. Algan O, Stobbe CC, Helt AM, Hanks GE, Chapman Jd. Radiation inactivation of human prostate cancer cells: the role of apoptosis. Radiat Res 1996;146:267–75.

2. Krajewska M, Krajewski S, Epstein Ji, et al. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol 1996;148:1567–76.

3. Rosser CJ, Reyes AO, Vakar-Lopez F, et al. Bcl-2 is significantly overexpressed in localized radio-recurrent prostate carcinoma, compared with localized radio-naive prostate carcinoma. Int J Radiat Oncol Biol Phys 2003;56:1–6.

4. Furuya Y, Krajewski S, Epstein JI, Reed JC, Isaacs JT. Expression of bcl-2 and the progression of human and rodent prostatic cancers. Clin Cancer Res 1996;2:389–98.

5. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997;388:300–4.

6. Gleave M, Nelson C, Chi K. Antisense targets to enhance hormone and cytotoxic therapies in advanced prostate cancer. Curr Drug Targets 2003;4:209–21.

7. Vilenchik M, Raffo AJ, Benimetskaya L, Shames D, Stein CA. Antisense RNA down-regulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents. Cancer Res 2002; 62:2175–83.

8. Leung S, Miyake H, Zellweger T, Tolcher A, Gleave ME. Synergistic chemosensitization and inhibition of progression to androgen independence by antisense Bcl-2 oligodeoxynucleotide and paclitaxel in the LNCaP prostate tumor model. Int J Cancer 2001;91:846–50.

9. Wacheck V, Selzer E, Gunsberg P, et al. Bcl-x(L) antisense oligonucleotides radiosensitise colon cancer cells. Br J Cancer 2003;89:1352–7.

10. Scott SI, Higdon R, Beckett L, et al. BCL2 antisense reduces prostate cancer cell survival following irradiation. Cancer Biother Radiopharm 2002;17:647–56.

11. Dan HC, Sun M, Kaneko S, et al. Akt phosphorylation and stabilization of XIAP. J Biol Chem 2003;M312044200.

12. Kitada S, Leone M, Sareth S, Zhai D, Reed JC, Pellecchia M. Discovery, characterization, and structure-activity relationships studies of proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins. J Med Chem 2003;46:4259–64.

13. Danial NN, Gramm CF, Scorrano L, et al. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis [see comment]. Nature 2003;424:952–6.

14. Zhang M, Liu H, Guo R, et al. Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. Biochem Pharmacol 2003;66:93–103.

15. Xu L, Pirollo KF, Chang EH. Transferrin-liposome-mediated p53 sensitization of squamous cell carcinoma of the head and neck to radiation *in vitro*. Hum Gene Ther 1997;8:467–75.

16. Usuda J, Chiu SM, Azizuddin K, et al. Promotion of photodynamic therapy-induced apoptosis by the mitochondrial protein Smac/DIABLO: dependence on Bax. Photochem Photobiol 2002;76:217–23.

17. Lawrence TS, Davis MA, Hough A, Rehemtulla A. The role of apoptosis in 2′,2′-difluoro-2′-deoxycytidine (gemcitabine)-mediated radiosensitization. Clin Cancer Res 2001;7:314–9.

18. Bischof M, Huber P, Stoffregen C, Wannenmacher M, Weber K-J. Radiosensitization by pemetrexed of human colon carcinoma cells in different cell cycle phases. Int J Radiat Oncol Biol Phys 2003;57:289–92.

19. Xu L, Pirollo KF, Tang WH, Rait A, Chang EH. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. Hum Gene Ther 1999;10:2941–52.

20. Xu L, Tang WH, Huang CC, et al. Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. Mol Med 2001;7:723–34.

21. Xu L, Frederik P, Pirollo KF, et al. Self-assembly of a virus-mimicking nanostructure system for efficient tumor-targeted gene delivery. Hum Gene Ther 2002;13:469–81.

22. Pirollo KF, Xu L, Chang EH. Non-viral gene delivery for p53. Curr Opin Mol Ther 2000;2:168–75.

23. Xu L, Huang CC, Huang W, et al. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. Mol Cancer Ther 2002;1:337–46.

24. Chou TC. Assessment of synergistic and antagonistic effects of chemotherapeutic agents *in vitro*. Contrib Gynecol Obstet 1994;19: 91–107.

25. Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr, Chou TC. Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. Cancer Res 1999;59:6178–84.

26. Takahashi N, Li W, Banerjee D, et al. Sequence-dependent synergistic cytotoxicity of ecteinascidin-743 and paclitaxel in human breast cancer cell lines *in vitro* and *in vivo*. Cancer Res 2002;62: 6909–15.

27. Chou TC, Otter GM, Sirotnak FM. Schedule-dependent synergism of taxol or taxotere with edatrexate against human breast cancer cells *in vitro*. Cancer Chemother Pharmacol 1996;37:222–8.

28. Tu Y, Stepkowski SM, Chou TC, Kahan BD. The synergistic effects of cyclosporine, sirolimus, and brequinar on heart allograft survival in mice. Transplantation 1995;59:177–83.

29. Chou TC, Tan QH, Sirotnak FM. Quantitation of the synergistic interaction of edatrexate and cisplatin *in vitro*. Cancer Chemother Pharmacol 1993;31:259–64.

30. Chou TC, Motzer RJ, Tong Y, Bosl GJ. Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design [comment]. J Natl Cancer Inst 1994;86: 1517–24.

31. Kruit A, Reyes-Moreno C, Newling DW, Geldof A, Koutsilieris M. Response of PC-3 prostate cancer cells to combination therapy using irradiation with glucocorticoids or doxorubicin. Anticancer Res 1999;19: 3153–6.

32. Ligueros M, Jeoung D, Tang B, Hochhauser D, Reidenberg Mm, Sonenberg M. Gossypol inhibition of mitosis, cyclin D1 and Rb protein in human mammary cancer cells and cyclin-D1 transfected human fibrosarcoma cells. British J Cancer 1997;76:21–8.

33. Han ML, Wang YF, Ma XH. A comparative study on rapid and slow loading of gossypol in the treatment of gynecological diseases. Acta Academiae Medicinae Sinicae 1984;6:270–2.

34. Palayoor ST, Bump EA, Teicher BA, Coleman CN. Apoptosis and clonogenic cell death in PC3 human prostate cancer cells after treatment with gamma radiation and suramin. Radiat Res 1997;148: 105–14.

35. Corbett TH. Transplantable syngeneic rodent tumors. Totowa: Humana Press; 2002. p. 41–71.

36. Blumenstein M, Hossfeld DK, Duhrsen U. Indirect radiation leukemogenesis in DBA/2 mice: increased expression of B2 repeats in FDC-P1 cells transformed by intracisternal A-particle transposition. Ann Hematol 1998;76:53–60.

37. Gleave ME, Zellweger T, Chi K, et al. Targeting anti-apoptotic genes upregulated by androgen withdrawal using antisense oligonucleotides to enhance androgen- and chemo-sensitivity in prostate cancer. Invest New Drugs 2002;20:145–58.

38. Inayat MS, Chendil D, Mohiuddin M, Elford HL, Gallicchio VS, Ahmed MM. Didox (a novel ribonucleotide reductase inhibitor) overcomes Bcl-2 mediated radiation resistance in prostate cancer cell line PC-3 [comment]. Cancer Biol Ther 2002;1:539–45.

39. Yurtcu E, Ergun MA, Menevse A. Apoptotic effect of gossypol on human lymphocytes. Cell Biol Int 2003;27:791–4.

40. Risco CA, Adams AL, Seebohm S, et al. Effects of gossypol from cottonseed on hematological responses and plasma α-tocopherol concentration of dairy cows. J Dairy Sci 2002;85:3395–402.

41. Cheng JS, Liu CP, Lo YK, et al. Gossypol, a component in cottonseed, induced increases in cytosolic Ca^{2+} levels in Chang liver cells. Toxicon 2002;40:851–6.

42. Herrera B, Fernandez M, Benito M, Fabregat I. cIAP-1, but not XIAP, is cleaved by caspases during the apoptosis induced by TGF-[β] in fetal rat hepatocytes. FEBS Letters 2002;520:93–6.

43. Van Poznak C, Seidman Ad, Reidenberg MM, et al. Oral gossypol in the treatment of patients with refractory metastatic breast cancer: a phase I/II clinical trial. Breast Cancer Res Treat 2001;66:239–48.

44. Mego M. Telomerase inhibitors in anticancer therapy: gossypol as a potential telomerase inhibitor. Bratisl Lek Listy 2002;103:378–81.

45. Benz CC, Iyer SB, Asgari HS, Matlin SA, Aronson FR, Barchowsky A. Gossypol effects on endothelial cells and tumor blood flow. Life Sci 1991;49:PL67-72.

46. Plesnila N, Zinkel S, Amin-Hanjani S, Qiu J, Korsmeyer SJ, Moskowitz MA. Function of BID—a molecule of the bcl-2 family—in ischemic cell death in the brain. Eur Surg Res 2002;34:37–41.

47. Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. Cancer Res 1994;54:1707–14.

48. Coyle T, Levante S, Shetler M, Winfield J. *In vitro* and *in vivo* cytotoxicity of gossypol against central nervous system tumor cell lines. J Neurooncol 1994;19:25–35.

49. Benz CC, Keniry MA, Ford JM, et al. Biochemical correlates of the antitumor and antimitochondrial properties of gossypol enantiomers. Mol Pharmacol 1990;37:840–7.

50. Oliver CL, Miranda MB, Wang S, et al. (−)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-XL-mediated apoptosis resistance. Mol Cancer Ther. In press 2005.

51. Mohammad RM, Wang S, Wu X, et al. Preclinical studies of (-)-Gossypol, a potent small molecule inhibitor of Bcl-2 and Bcl-XL, against diffuse large cell lymphoma (DLCL) xenograft model. Mol Cancer Ther. In press 2005.

52. Kyprianou N, King ED, Bradbury D, Rhee JG. Bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells. Int J Cancer 1997;70: 341–8.

53. Gutierrez-Puente Y, Zapata-Benavides P, Tari AM, Lopez-Berestein G. Bcl-2-related antisense therapy. Semin Oncol 2002;29:71–6.

54. Shangary S, Johnson DE. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. Leukemia 2003;17:1470–81.

55. Tolcher AW. Preliminary phase I results of G3139 (bcl-2 antisense oligonucleotide) therapy in combination with docetaxel in hormone-refractory prostate cancer. Semin Oncol 2001;28:67–70.

56. Davies AM, Gandara DR, Lara PN Jr., Mack PC, Lau DH, Gumerlock PH. Antisense oligonucleotides in the treatment of non-small-cell lung cancer. Clin Lung Cancer 2003;4 Suppl 2:S68-73.

57. Guensberg P, Wacheck V, Lucas T, et al. Bcl-xL antisense oligonucleotides chemosensitize human glioblastoma cells. Chemother 2002;48:189–95.

58. Wacheck V, Losert D, Gunsberg P, et al. Small interfering RNA targeting bcl-2 sensitizes malignant melanoma. Oligonucleotides 2003;13:393–400.

59. Oliver CL, Bauer JA, Ubell ML, et al. *In vitro* effects of the BH3 mimetic, (−) Gossypol, on head and neck squamous cell carcinoma cells. Clin Cancer Res. 2004;10:7757–63.

60. Del Bufalo D, Trisciuoglio D, Scarsella M, Zangemeister-Wittke U, Zupi G. Treatment of melanoma cells with a bcl-2/bcl-xL antisense oligonucleotide induces antiangiogenic activity. Oncogene 2003;22: 8441–7.

61. Fernandez A, Udagawa T, Schwesinger C, et al. Angiogenic potential of prostate carcinoma cells overexpressing bcl-2. J Natl Cancer Inst 2001;93:208–13.